2.5 Healthcare Measurements

Chemical metrology is at the heart of accurate medical diagnosis and the development of measures to improve our health and ensure long life. In the U.S. about 1.5 trillion dollars are spent each year on health care, which is over 14 % of our GDP. More than 25% of these expenditures are for measurements. It is estimated that over one third of these measurements are performed for non-diagnostic purposes, such as QA/QC measurements and retests at a cost of about $40B annually. Clearly, improvements in the reliability of chemical measurements in this area would have a significant economic impact for our country.

NIST/CSTL works closely with the American Association for Clinical Chemistry (AACC), the Centers for Disease Control and Prevention (CDC), NCCLS, and other organizations interested in health-related standards to help prioritize our standards activities and the development of SRMs.

CSTL's Healthcare Measurements Program addresses measurements and standards needs of the medical industry – in the areas of prevention, diagnostics, and treatment.

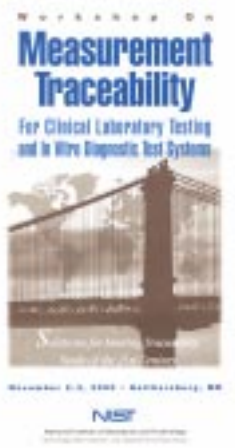
For more than 20 years CSTL has developed, maintained, and refined "Definitive Methods" for 12 health status markers to support the national reference system for clinical measurements: calcium, chlorine, cholesterol, creatinine, glucose, lithium, magnesium, potassium, sodium, triglycerides, urea, and uric acid. NIST definitive methods for these health status indicators have been used to value-assign SRMs and high-priority serum pools used to serve as the anchor point for CDC developed reference methods and by the College of American Pathologist (CAP) for proficiency testing of more than 20,000 U.S. clinical laboratories. Improved accuracy facilitated by this program has led to better diagnosis, treatment, and reduced healthcare costs. Maintaining these anchor points for the clinical measurements reference system also facilitates the development and use of new technologies that are better, faster and cheaper.

A new generation of health status markers, emerging now, shows great promise from the clinical diagnostic perspective, but offers new and more difficult challenges for standardization. Many of the new markers are proteins, peptides, or other large biomolecules, usually present at very low concentrations. Because of the large market for tests for these new markers, many different approaches have been developed commercially resulting in vast disagreements among manufacturer test kits. NIST/ CSTL has focused on basic research to establish reference systems for several new biomarkers, shown in the box to the right.

• **Troponin**	***heart attack marker***
• **Homocysteine**	***risk of heart disease***
• **Glycated Hemoglobin**	***diabetes status***
• **Cortisol**	***endocrine function***
• **Thyroxine**	***thyroid function***
• **Cadmium**	***heavy metal toxicity***
• **Folic Acid**	***neural tube defects***
• **Mercury**	***heavy metal toxicity***
• **Speciated Iron**	***hemochromatosis, anemia***
• **Human Serum Albumin**	***renal failure***
• **Prostate Specific Antigen**	***prostate cancer***
• **P53 DNA**	***breast cancer***
• **Thyroid Stimulating Hormone**	***thyroid function***

Another driving force for more clinical reference standards is the new European Community (EC) *In Vitro* Diagnostic Devices (IVDD) directive that requires traceability of IVD devices to recognized national standards. By December 2003, all IVD products sold in Europe must have the "EC Mark" verifying compliance with the directive. U.S. manufacturers are major exporters of IVD products and thus are directly impacted by this directive.

In November 2000, NIST hosted a workshop on Measurement Traceability for Clinical Laboratory Testing and *In Vitro* Diagnostic Devices. CSTL worked closely with the workshop cosponsors, NCCLS, AACC, AdvaMed (formerly HIMA), CDC, and College of American Pathologists (CAP) to ensure that the needs of the IVD industry were presented and met. The workshop goals were to develop recommendations regarding the needs for measurement traceability for health status markers to (1) address IVD industry needs for compliance with international standards (e.g., EU IVD Directive) and (2) improve comparability of clinical measurement data to facilitate better decision making by medical professionals.



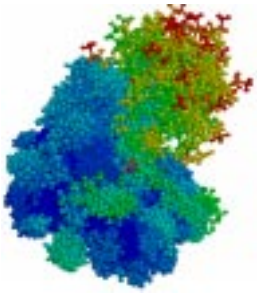
The status of current health status marker work, grouped by disease state, is described below.

Markers to Improve Disease Diagnosis and Management

Cardiac Troponin-I (cTnI): Marker to Diagnose Heart Disease

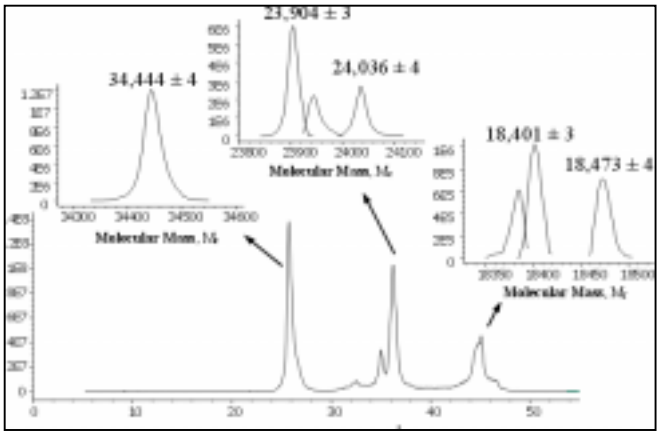
D. Bunk, J. Dalluge, M. Welch (839), R. Christenson, and S.H. Duh (University of Maryland)

The measurement of cTnI in serum provides a highly selective and sensitive means for diagnosing myocardial infarction. For clinical cTnI measurement, there are numerous cTnI assays that have been developed. Unfortunately, cTnI measurements using different methods on identical samples may differ by 100-fold, creating a serious problem for the clinical community. The Troponin-I subcommittee chose ten candidate reference materials, which were analyzed at NIST for purity and structural heterogeneity using liquid chromatography coupled with mass spectrometry (LC/MS). The candidate reference materials were sent to all manufacturers of commercial cTnI assays used in the United States and Europe (13 manufacturers in total) for analysis using their cTnI assays.



NIST, the American Association for Clinical Chemistry (AACC), and the International Federation of Clinical Chemistry (IFCC) have formed a subcommittee to address inter-method variability problems in clinical cTnI measurement, through development of a cTnI reference material to harmonize results.

Results from these analyses were collected and subjected to statistical analysis that found that two candidate reference materials, both complexes of troponin I with other troponin isoforms, provided the most linear response for all thirteen cTnI assays. The next



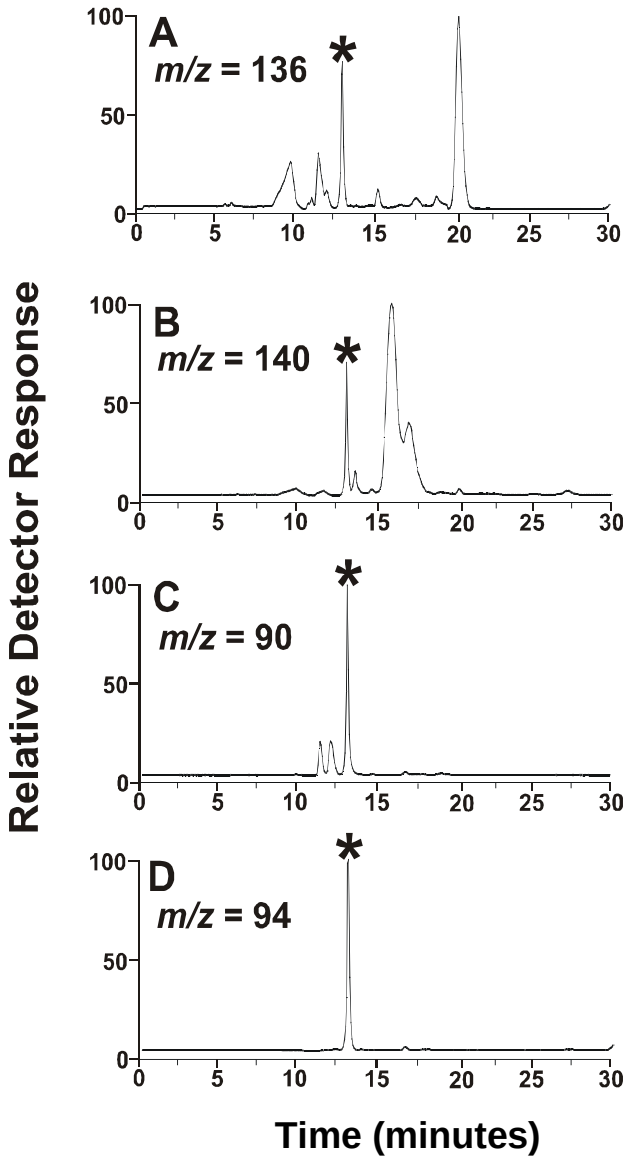
Total ion chromatogram from the LC/MS analysis of the cardiac troponin CIT complex from the University of Miami. The inserts show the molecular mass distributions from the troponin T, troponin I, and troponin C peaks, respectively.

comparison study will involve these two materials and blood samples from patients with myocardial infarction. The candidate reference materials will be used to calibrate the commercial cTnI assays prior to measuring the patient samples. This study should determine the material that will best harmonize the commercial cTnI assays.

Homocysteine: Marker to Diagnose Heart Disease

J. Dalluge, B. Nelson, L.T. Sniegoski, and S. Margolis (839)

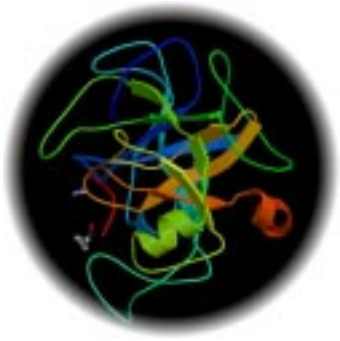
Total homocysteine (tHcy) has emerged as an important independent risk factor for cardiovascular disease, as well as other serious health conditions. tHcy is measured clinically using a variety of methods including immunoassays and LC-based methods requiring extensive derivatization procedures and frequent analysis of quality control samples. Because clinical laboratories use a variety of different methods for its measurement, interlaboratory comparisons of tHcy measurements are poor. Work at NIST has focused on development of LC/MS and GC/MS methods. For the LC/MS method, chromatographic columns and elution conditions were investigated to achieve retention of homocysteine. This led to development of an elution system that allowed unambiguous detection of two homocysteine-specific ions and two isotopically labeled homocysteine-specific ions (internal standard) in human plasma. The quantitative capabilities of this approach will be tested in conjunction with the GC/MS method under development and in collaboration with scientists at the Mayo Clinic. Use of GC/MS requires additional separations and derivatization prior to analysis. An anion exchange method was found that provided the necessary analyte isolation. Thus, the GC/MS method should be ready for further testing in the near future. Once the methods have been validated, they will be applied to the determination of homocysteine in plasma-based reference materials.



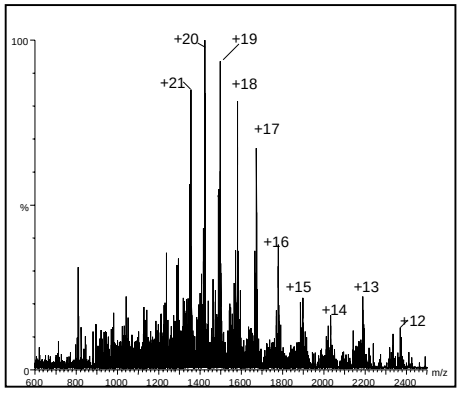
Identification of Hcy and d_4 –Hcy in human plasma using LC/MS. The peaks corresponding to Hcy and d_4 –Hcy in the mixture are labeled with an asterisk. Concentration of Hcy and d_4 –Hcy in the plasma sample was 15 µmol.

Prostate-Specific Antigen (PSA): Marker to Diagnose Cancer

C. Nelson, J. Dalluge, D.M. Bunk, and M.J. Welch (839)



PSA is a single chain glycoprotein (~ 7% - 8% carbohydrate) present in the prostate and seminal fluid. The presence of PSA in the serum is measured as a diagnostic indicator of prostatic cancer. Clinical laboratories currently measure PSA by immunoassays, which are confounded by lack of common standards, and the heterogeneity of the antisera and antigen being measured. Measurement of PSA is a significant challenge due to the structural heterogeneity of PSA in serum, its low concentration in this complex matrix (<10 ng/mL), and lack of knowledge regarding what form is actually being measured in clinical PSA tests. Current research efforts are focused on characterization of a variety of PSA standards at the molecular level by LC/MS in order to understand further the heterogeneity of the analyte, arising predominately from differential lycosylation of the protein. Reproducible full scan (m/z 600 to 2500) positive charged mass spectra were obtained with a commercial source of PSA. Deconvolution of the mass spectrum showed a predominant molecular species at M_r 28,447; the predicted M_r for the glycosylated mature PSA is 28,430. Selected ion monitoring of specific positive charge states was utilized to detect PSA standards at levels as low as 50 ng. Further efforts will focus on: 1) production of a well-characterized SRM for improvement of between-method variations in calibration of immunoassay measurements, 2) determination of specific forms of PSA in patient samples to understand better the clinically relevant forms of this compound, 3) isolation of PSA from serum using non-immunological methodology including combinatorial production of aptamers with high binding affinities for PSA, 4) development of LC/MS and LC/MS/MS methods for the measurement of PSA in serum.

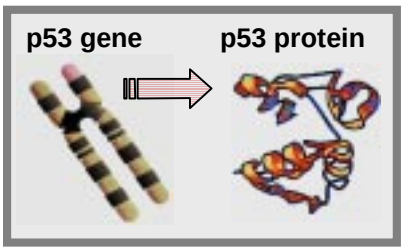


Full scan mass spectrum of prostate-specific antigen obtained by liquid chromatography/electrospray ionization mass spectrometry

Evaluation of p53 Standard Reference Materials Using Mutational Scanning Technologies: Marker to Diagnose Cancer

C.D. O'Connell, B.S. Akbasak, L. Tully, D.H. Atha (831), J. Delaney, and M. Marino (Transgenomic, Inc.)

We have created a panel of p53 mutation standards for use in mutation detection technologies as part of our molecular diagnostic measurements program. These materials were used as positive controls to assess the mutational status of clinical specimens. Although DNA sequencing remains the "Gold Standard" for the detection of mutations, a number of mutational scanning technologies are being developed to reduce the region of the gene that needs to be sequenced, thus saving both time and cost. Measurement standards are required to accurately compare these mutation detection technologies.



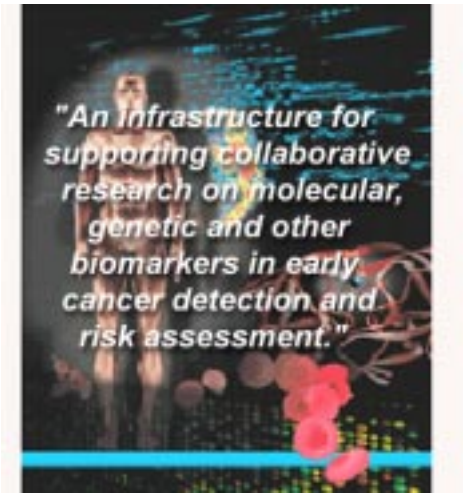
Measurements on clinical specimens were performed using proposed NIST SRM materials. Archival clinical samples were evaluated for p53 mutations within exons 5 through 9, the most commonly mutated region of this gene. The NIST materials, used for both positive and negative mutational controls, consisted of 11 mutant and 1 wild-type clone.

Four different mutation detection technologies were used in this study: 1) DNA sequencing, 2) single strand conformation polymorphism analysis (SSCP), 3) denaturing gradient gel electrophoresis (DGGE), and 4) denaturing high performance liquid chromatography (DHPLC).

The two heteroduplex-based methods (DGGE and DHPLC) yielded identical results: 6 mutations were detected in the 33 samples analyzed. These were confirmed by DNA sequencing. Difficulties in gel-based separation (DGGE) of exon 5 heteroduplexes were not observed in the DHPLC measurements. All but one mutation detected by heteroduplex analysis were also observed by slab gel and capillary SSCP measurements. Multiple conformers were detected by SSCP in both slab- and capillary-based measurements, consistent with previous reports using this technology. The presence of multiple conformers representing a single species of DNA (wild-type or mutant) is a complication of the SSCP assay system. The wild-type standard was important in distinguishing wild-type conformers from mutations. Both slab gel SSCP and capillary SSCP detected additional conformers with respect to wild type in samples not reported as mutant by heteroduplex analysis. Automated fluorescent DNA sequencing did not confirm these putative mutations. These "mutations" may be present at too low a percentage in the mixed population of normal and tumor tissue to be detected by sequencing or heteroduplex analysis. Alternatively, additional conformers representing wild-type DNA are formed in the SSCP analysis of clinically derived genomic DNA samples. Future studies are aimed at determining the detection limits of these mutational scanning technologies for the identification of mutations in heterogeneous clinical materials.

NIST Biomarker Validation Laboratory

P.E. Barker and C.D. O'Connell (831)



As a collaboration with the National Cancer Institute's new consortium The *Early Detection Research Network* (EDRN), CSTL's Biotechnology Division was selected to serve as the ***Biomarker Validation Laboratory*** for new cancer detection biomarkers discovered in this network. This 5-year, $2.2 million Interagency Agreement will focus on biomarkers with a strong nucleic acid component that are well within the expertise and interests of the DNA Technologies Group. Projects approved by the EDRN for validation include a project "Validation of the mutagen sensitivity and chromosomal hotspot assays" to be performed in collaboration with M.D. Anderson Cancer Center. Other biomarkers still in approval stage include assays for mtDNA sequence changes in head and neck tumors and serum levels of the enzyme telomerase.

Thyroid-Stimulating Hormone and Thyroxine: Marker to Diagnose Thyroid Disease

S.S. Tai, L.T. Sniegowksi, and M.J. Welch (839)

Human thyroid-stimulating hormone (TSH) is a hormone secreted by the pituitary gland. Its role is in the stimulation of the thyroid gland to produce the thyroid hormones triiodothyroxine (T3) and thyroxine (T4). Serum TSH levels are used as a diagnostic tool for assessing thyroid function. Increased TSH levels can be indicative of hypothyroidism, a condition in which the thyroid gland fails to recognize TSH and decreases its production of T3 and T4. TSH is structurally heterogeneous and present at very low levels in human serum, and therefore presents a significant measurement challenge. Work to date has focused on developing approaches for characterizing TSH preparations. We found that treating TSH with sialidase to remove sialic acid residues from the carbohydrate moieties on these proteins significantly improved their mass spectrometric characterization. Full characterization of the TSH preparations will require peptide mapping to verify identity and primary structure and characterization of carbohydrate heterogeneity.

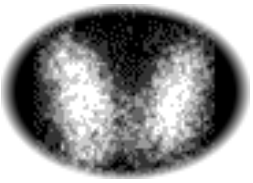
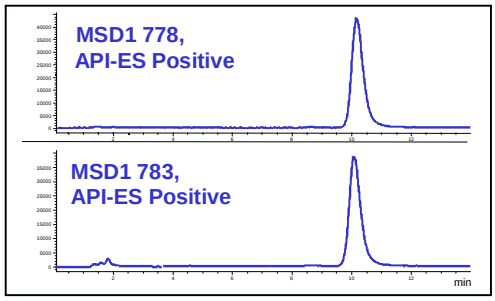


Image of a Normal Thyroid

Thyroxine is a hormone secreted by the thyroid gland that is a stimulator for a number of functions and is important in growth, development, and sexual maturation. The concentration of total thyroxine in serum is about 50 ng/mL - 110 ng/mL, and is used as a measure of thyroid function. A new method based on isotope dilution liquid chromatography/mass spectrometry using electrospray for ionization (LC/MS-ESI) has been developed for the determination of total thyroxine in serum. Samples of CAP survey sera were prepared and measured on three separate sets. Excellent precision was obtained for all three levels of serum samples with within-set CVs ranging from 0.2% to 1.0%. Excellent linearity was obtained with the correlation coefficients of all linear regression lines ranging from 0.999 to 1.000. Positive and negative ion measurements agree within 0.8%. The detection limit at a signal to noise ratio of approximately 3 to 5 for thyroxine with this method is estimated to be 30 pg and 20 pg for positive and negative ion, respectively. The LC/MS-ESI method was tested against field methods (about 1900 laboratories from CAP surveys). The results of the LC/MS-ESI method and field methods compared well with an average difference of 5% for all three levels. This method will be used to measure the thyroxine level in some existing serum-based SRMs. Future plans are to develop a new SRM with both low and elevated levels of thyroxine and cortisol. More than 99.9% of thyroxine in blood is bound to protein. There is considerable interest in measuring free thyroxine, but clinical methods give widely varying results. The isolation of the free thyroxine and its measurement by LC/MS will be investigated.



Single Ion Chromatograms by LC/MS-ESI for Thyroxine and Thyroxine-d_5 from a Serum Sample

Glycated Hemoglobin: Marker for Improved Management of Diabetes

D. Bunk and J. Dalluge (839)

Approximately 16 million people in the United States have diabetes, with nearly 2,200 new cases diagnosed each day. About 95% of the people with diabetes have type II disease.

Glucose accumulates in the blood of those with type II produce insulin since the cells in their bodies are "insulin resistant" and do not respond properly to the hormone. Left untreated, diabetes can result in cardiovascular disease, kidney disease, eye diseases, and nervous-system maladies. In addition to the daily monitoring of blood glucose levels, the measurement of glycated hemoglobin (HbA_{1c}) plays an important role in the diagnosis and treatment of diabetes. When blood glucose levels rise above normal, the glucose can react with hemoglobin in the blood, forming HbA_{1c}. Since the lifetime of hemoglobin in the bloodstream is approximately 3 to 4 months, the measurement of HbA_{1c} provides a record of the levels of blood glucose over the course of 3 to 4 months. Glycated hemoglobin measurement provides doctors with information on the efficacy of diabetes treatment over a longer period than daily blood glucose measurements. While the medical utility of HbA_{1c} measurement has been clearly demonstrated, the clinical application of HbA_{1c} measurement has demonstrated considerable problems with method-to-method variability. There is a strong need for HbA_{1c} measurement standardization. The International Federation for Clinical Chemistry (IFCC) has developed two reference methods for the determination of HbA_{1c} in blood hemolysates. One method uses liquid chromatography coupled with mass spectrometry (LC/MS) and the other used capillary electrophoresis (CE) for HbA_{1c} determination. Both methods have demonstrated high precision (average inter-laboratory coefficient of variance $\leq$ 2%) and excellent agreement with each other. These reference methods have been implemented in a network of laboratories worldwide. Currently the IFCC reference methods are being used to value-assign an international HbA_{1c} reference material

NIST has participated in inlaboratory comparison studies of IFCC reference materials and has provided critiques for improvements.

Iron-Containing Proteins: Marker for Diseases Related to Abnormal Serum Iron Levels

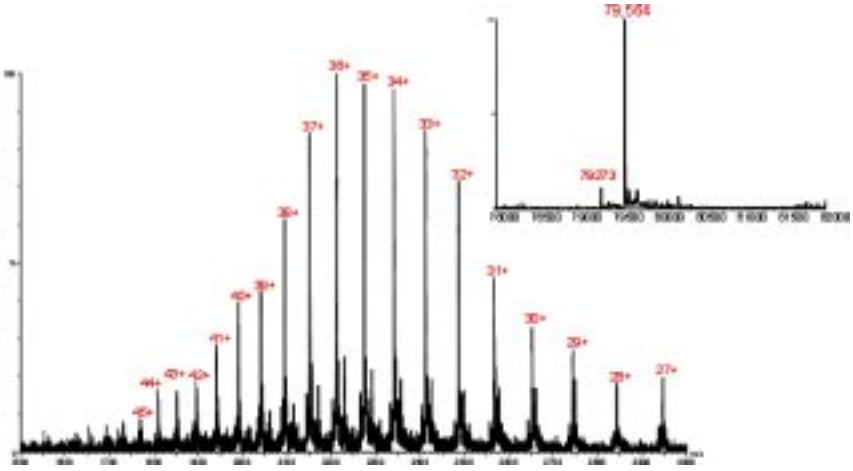
D. Bunk and S.E. Long (839)

Serum iron determinations are an important clinical measurement. According to the CDC hereditary hemochromatosis is the most common genetic disorder in the United States, with approximately one in one hundred people at risk for developing the disease. Typically, for the purpose of medical diagnosis, the total serum iron concentration is measured. However, in many iron-related diseases, the distribution of iron bound to serum proteins is altered. For improved medical diagnosis, it would be beneficial to determine the distribution of iron among serum proteins as well as the total serum iron concentration. Work has begun on development of methodologies that will lead to a reference material for speciated iron in serum, a material that could be used for the more routine clinical measurements of total serum

Low serum iron levels **are indicative of diseases such as anemia, rheumatoid arthritis, and certain infections.**

Hemochromatosis, the most common form of *iron overload disease*, is an inherited disorder that causes the body to absorb and store too much iron, often resulting in failure of the liver, heart, or pancreas.

Transferrin
Molecular Mass = 75,143



Human Transferrin (Tf)
Electrospray-ionization mass spectra

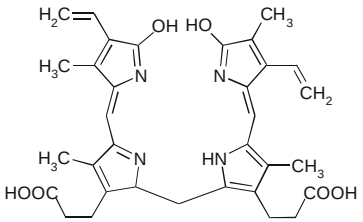
iron, but also used for the more advanced diagnosis of iron-related diseases. The two important iron-containing proteins in human serum are transferrin and ferritin. The concentration of ferritin in serum is approximately one thousand times lower than that of transferrin. However, medical research is indicating that serum ferritin determination can often be a more selective diagnosis tool than determination using transferrin. Development of a quantitative method for serum transferrin using liquid chromatography coupled with mass spectrometry (LC/MS) and affinity chromatography is underway. The concentration of iron associated with the transferrin will be measured using high-resolution inductively coupled plasma mass spectrometry (ICP/MS). Together, the LC/MS and the ICP/MS measurements provide highly selective and high-precision values for both serum transferrin and serum iron levels.

Bilirubin: Marker for Elevated Liver Function

Y.Y. Davidson, L.T. Sniegoski, and M.J. Welch (839)

Elevated levels of bilirubin in blood are indicative of impaired liver function.

Bilirubin, the orange-yellow bile pigment, is produced from protoporphyrin IX by microsomal heme oxygenase. There are three principal isomers of bilirubin, which may be free or complexed with other blood constituents. Clinical laboratory measurements of bilirubin exhibit considerable variability because of method differences and calibration errors. Our goal is to develop an LC/MS reference method to measure bilirubin. Efforts have focused on first establishing the spectrophotometric reference method based upon a direct diazo reaction. Plans are to use this method as a tool for validation of the LC/MS method, and then to use both methods for certification of bilirubin in serum-based SRMs.



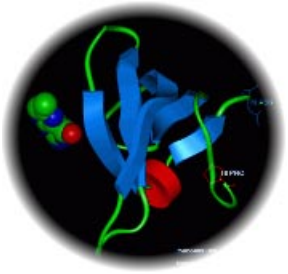
Folic Acid: Marker for Neural Tube Defects

B.C. Nelson, J.J. Dalluge, S.A. Margolis, D.Z. Bezebeh, and L.C. Sander (839)

Folic acid is a water-soluble B vitamin that plays a significant role in human health:

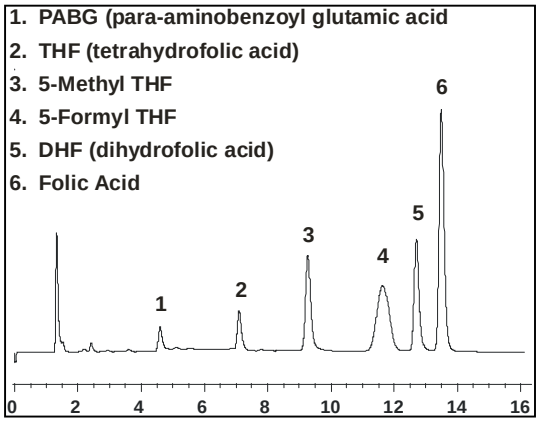
1) A deficiency of folic acid during gestation causes neural tube malformations that result in spina bifida.

2) A deficiency of folic acid causes the accumulation of high levels of amino acid homocysteine that has been linked to the increased risk of cardiovascular disease and other diseases.



Folic Acid Binding Protein

The ability to determine folic acid status accurately is a significant challenge due to the existence of up to eight metabolites, many of which are unstable, and their low levels in plasma and serum (total folate concentration ≤ 20 ng/ml). A gradient elution liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) method for the separation and detection of the five most biologically relevant folates has been developed and applied to the quantitation of folates in human plasma/serum. The method involves the *in vitro* stabilization of plasma samples via a dual antioxidant system (*L*-ascorbic acid/*L*-cysteine), protein precipitation and sample concentration. The major circulating folate (5-methyl tetrahydrofolic acid) in human plasma/serum is detectable and quantifiable based on a standard additions procedure using an endogenous plasma component as an internal standard. The other biologically relevant folates are not detectable in normal human plasma. This group of researchers is also developing a second method based on the use of liquid chromatography with coulometric detection for the determination of folates in plasma. Current efforts are focused on developing a folate quantitation procedure based on the use of external standard calibrants so that the LC/MS method might be more adaptable to routine clinical use.



Diagnostic and Therapeutic Standards

Lead, Cadmium, Total Mercury, and Methyl Mercury

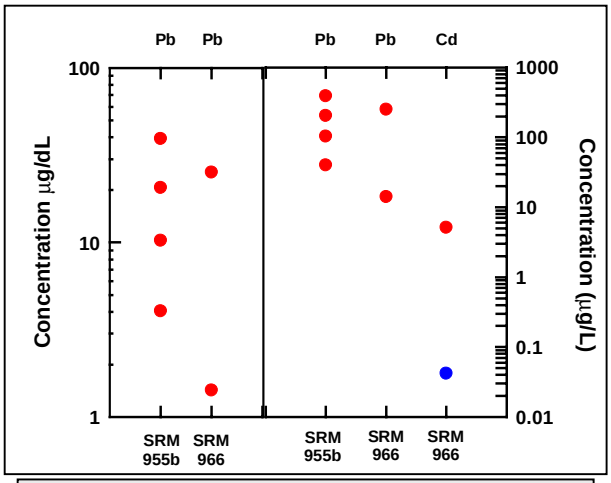
S.E. Long, M.S. Rearick, R.D. Vocke, and E.A. Mackey (839)

A whole blood reference material, SRM 966, for evaluating the accuracy of lead (Pb), cadmium (Cd), total mercury (Hg) and methyl mercury (MeHg) measurements in whole blood at natural and slightly elevated levels has been released this year. Analysis of whole blood is one of the most common ways exposure to these toxic trace elements is monitored in the human population.

New OSHA workplace monitoring standards now require measurements of blood cadmium; prior to this SRM, only proficiency samples with consensus cadmium values existed as "standards".

Also, a large number of organizations in the human health monitoring area (e.g. CDC, NIEHS, EPA, and WHO) have indicated their interest in biological SRMs (especially blood) certified for MeHg. A unit of this SRM consists of two vials from each of two levels (natural and elevated), containing approximately 2 mL of whole blood each. The bovine blood base material was prepared for NIST at a USDA licensed facility from cows bled after dosing with lead nitrate.

Endogenous Pb concentrations were certified by isotope dilution (ID) ICP-MS, a primary method for this analyte in clinical materials. Cd concentrations in the elevated level were certified by combining data results from two independent methods, instrumental neutron activation analysis (INAA) and ID ICP-MS. Total Hg was certified in the elevated material by combining data from ID ICP-MS and cold vapor atomic absorption spectrometry (CVAAS). This material will support workplace and general public health monitoring for lead, cadmium, total mercury and MeHg in blood.



SRM 966 released in FY 2000 is a new addition to the NIST SRMs for Pb and Cd in Whole Blood

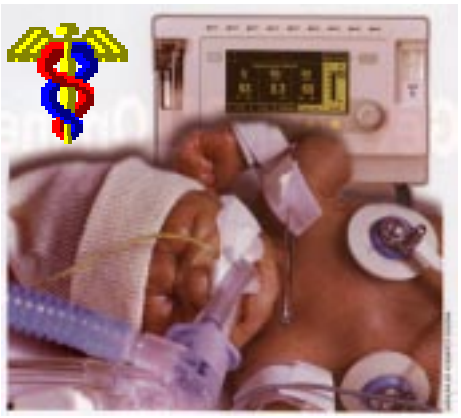
Inhaled Nitric Oxide Therapy

W.J. Thorn (839)

CSTL's work facilitates the implementation of Inhaled Nitric Oxide (INO) Therapy that could help save the lives of 2,000 U.S. newborns annually.

For more than five years, researchers in CSTL's Analytical Chemistry Division have been working with the National Insitute for Child Health and Development (NICHD) to facilitate the implementation of Inhaled Nitric Oxide (INO) Therapy. Early work involved the investigation of the kinetics of NO_2 formation from the NO used in INO. Dilute concentrations (<100 µmol/mol) of nitric oxide have shown dramatic results as a pulmonary vasodilator in some newborn patients when inhaled in oxygen via a ventilator. However once the nitric oxide is mixed with oxygen, the clock starts for the spontaneous formation of nitrogen dioxide. Accurate measurement of nitrogen dioxide is important because nitrogen dioxide (>5 µmol/mol) is considered detrimental - possibly leading to pulmonary edema and other negative consequences. Based on the NIST data it was concluded that at anticipated dwell times of <0.5 s, no significant levels of the harmful NO_2 should reach the patient during treatment.

At a workshop held at NIST last year, several of both types of instruments used for NO/NO_2 monitoring (chemiluminescence-based and electrochemical-based devices) were found to measure NO and NO_2 inaccurately under high oxygen conditions. NIST developed the primary standards that provide the accuracy base for these measurements and support the production of the necessary standards by a commercial specialty gas company of NTRM gas mixtures that are used to calibrate these monitoring devices. At the request of the NICHD NIST developed a NIST Traceable Reference Material (NTRM) at 85 µmol/mol NO in nitrogen.



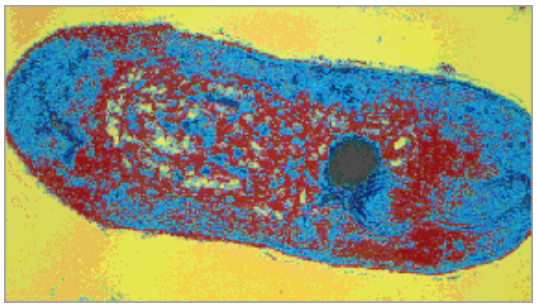
The methodology developed at NIST to simulate and deliver INO ventilator circuit mixtures of known concentrations was also used to evaluate the accuracy of redesigned monitoring devices in measuring NO and NO_2 and also was used to investigate possible biases due to oxygen quenching.

Based on the NIST research, nitric oxide, which helps babies breathe easier, was approved as a drug by the FDA in December 1999. Currently, NIST and the FDA are working with doctors and instrument manufacturers to develop written protocols and standards so that new INO devices can gain FDA approval. Once the devices are approved it is expected that this therapy will be commonplace in hospital neonatal care units.

NIST's role now is to serve on ASTM subcommittee F29.1.11 working with FDA anesthesiologists, medical doctors, and INO medical device manufacturers on a device acceptance "standard" which when followed will facilitate FDA device approval. NIST provides input to NO and NO_2 performance tests for gauging accuracy and the negative effects of interfering anesthesia gases.

Cloning, Expression, and Characterization of Chorismate Mutase from Mycobacterium Tuberculosis

P. Reddy (831)



Mycobacterium tuberculosis genome sequence revealed a gene for chorismate mutase. Chorismate mutase is present only in bacterial and lower eukaryotic systems but is absent in higher eukaryotes. Hence, this enzyme is a popular target in metabolic engineering and drug development. It is intriguing to determine the biochemical properties and three-dimensional structure of chorismate mutase in *M. tuberculosis*.

Chorismate mutase I gene was amplified by polymerase chain reaction (PCR). The primers for PCR amplification included NdeI and BamHI restriction endonuclease recognition sequences at the 5' and 3' ends, respectively. Amplified product was digested with the restriction enzymes, and the gene was cloned into a similarly digested pRE1 protein expression vector. A recombinant plasmid was introduced into *E. coli* strain MZI for protein expression. Expressed chorismate mutase was purified to homogeneity, and the enzymatic properties were studied.

Chorismate mutase catalyzes the pericyclic rearrangement of chorismate to prephanate, which can be converted to either tyrosine or phenylalanine. We investigated the functional nature of chorismate mutase from *M. tuberculosis*. Chorismate mutase is a 199 amino acid protein with an amino terminal signal sequence that is cleaved from the mature protein. The role of the signal sequence remains to be investigated. The enzyme has no associated activity for either prephanate dehydratase or prephanate dehydrogenase. Therefore, chorismate mutase from *M. tuberculosis* belongs to monofunctional class mutases. Preliminary structural characterization revealed that this enzyme belongs to monofunctional chorismate mutases but differs in structural diversity having only alpha helices in contrast to *B. subtilis* chorismate mutase which is also monofunctional containing alpha helices and beta strands.

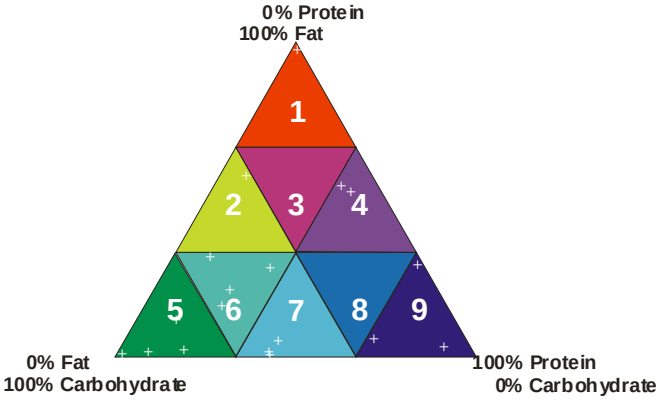
We will determine the three dimensional structure of chorismate mutase from *M. tuberculosis,* and design small molecule inhibitors, and test for inhibition of chorismate mutase activity. This research will revolutionize drug discovery for *M. tuberculosis.*

Nutritional Standards

Methods and Standards to Support Nutritional Labeling of Food Products

K.E. Sharpless, J. Brown Thomas, S.A. Margolis, B.C. Nelson, C.S. Phinney, and L.J. Wood (839)

The Nutrition Labeling and Education Act requires that specific nutritional information be provided on all processed foods sold in the U.S. In a 1996 study by the U.S. Food and Drug Administration, the accuracy of label information ranged from 98% to 54% for nutrients for which labeling is required. As more food-matrix SRMs become available, label accuracy should improve when the food and nutrition communities employ these SRMs in their analyses.



Association of Official Analytical Chemists (AOAC) International has developed a nine-sectored triangle in which foods are positioned based on their fat, protein, and carbohydrate content. AOAC's belief is that one or two reference materials within each sector should be representative of other foods within that sector and could be used for quality assurance and method validation when analyzing those other foods.

New Food SRMs in the Certification Process for FY 2000

SRM 2384 Baking Chocolate – Sector 2 – highest priority
The first reference material available from NIST with values assigned for caffeine, theobromine, and catechins.

SRM 2385 Spinach – Sector 7
To replace existing freeze-dried, finely ground SRMs with more natural material.

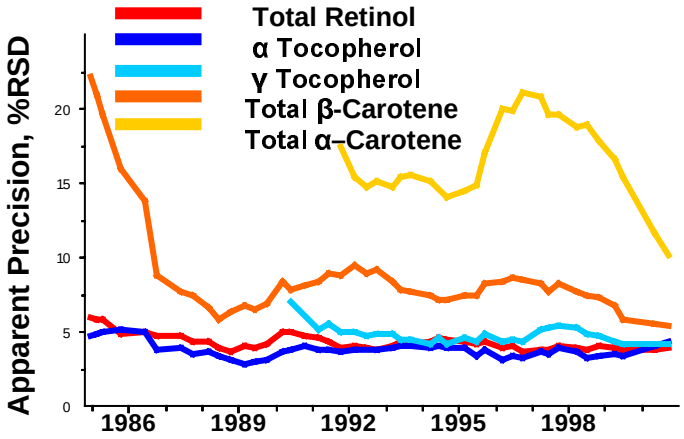
SRM 1946, Lake Superior Fish Tissue – Junction of sectors 4,8, and 9
In addition to the analytes of nutritional interest, this material will be certified for toxic trace metals, polychlorinated biphenyls, pesticides, and methylmercury.

High priority needs that have been identified as a result of a workshop included SRMs for aflatoxins and allergens such as peanut protein. (Foods must be labeled if they contain – or may contain – unexpected allergens. Permissible levels of aflatoxins are also regulated.) A peanut butter SRM would address the needs for aflatoxin and allergenic peanut protein reference materials as well as providing a reference material in sector 3 of the AOAC triangle, which is not occupied by any other SRMs.

The Micronutrients Measurement Quality Assurance Program

J. Brown Thomas, D.L. Duewer, S.A. Margolis, K.E. Sharpless (839), and M.C. Kline (831)

The Micronutrients Measurement Quality Assurance (QA) Program was organized to support measurement technology for selected fat- and water-soluble vitamins and carotenoids in human serum and plasma. It was initiated in 1984 as part of investigations supported by the National Cancer Institute (NCI) Division of Cancer Prevention and Control to study the possible role of these analytes in reducing the risk of developing certain types of cancers and diseases. Today it is the "the only QA program available for the fat-soluble vitamins and carotenoids" and currently includes more than 60 laboratories worldwide.



Micronutrients Measurement QA Program Comparison Exercise (from 1986 – 2000)

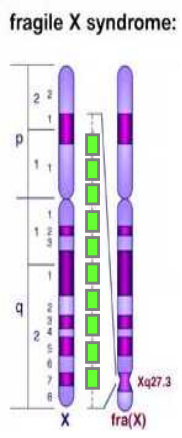
NIST provides laboratories with the tools for comparability assessment through use of interlaboratory comparison studies, Standard Reference Materials (SRMs) and control materials, and methods development and validation. Serum-based samples with assigned values for the target analytes and performance-evaluation standards are distributed by NIST to laboratories for analysis. NIST staff provide the laboratories with technical feedback concerning their performance as well as suggestions for methods development and refinement. The results from the comparison studies are used to establish a laboratory performance database, which is used to help laboratories to improve their measurement comparability and to obtain reliable data needed to make accurate clinical and health-care decisions

As a result of the QA program, the accuracy of laboratory measurements resulting in increased interlaboratory comparability for retinol, α-tocopherol, and β-carotene has improved substantially over time (see figure). The average estimated coefficient of variation for retinol and α-tocopherol has been approximately 5% for the past five years and about ≤10% for β-carotene for that same period of time.

DNA-Based Measurements

Standardization of Trinucleotide Repeat Measure

K.L. Richie, D.H. Atha, and C.D. O'Connell (831)

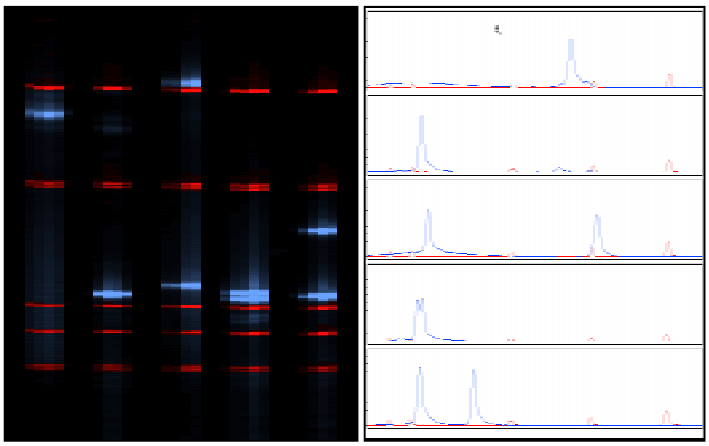


CSTL provides the clinical diagnostics community with accurate protocols and measurements for the detection of genetic disease. The Fragile X disease system used in this study requires the accurate quantitation of triplet repeats that confer important diagnostic information.

In this study, we focused on the triplet repeat causing Fragile X Syndrome for the following reasons. First, it is one of an increasing number of triplet repeat expansions associated with disease. Expansion of these repeat elements results in the interruption of gene expression and disease. Interruption of the FMR-1 gene occurs when more than 200 copies of the triplet repeat are present. Mental retardation is associated with this interruption in virtually all males with over 200 copies of the repeat, and females are affected to a lesser degree. Moreover, a "premutation"

state can be detected when 50 to 200 copies of the repeat are present. Secondly, Fragile X is the leading heritable cause of mental retardation; because it is inherited, accurate quantitation of the number of repeat sequences can be used for carrier screening in family planning in addition to diagnosis of disease and pre-natal screening. As such, we feel that our research will have the largest impact on diagnostic testing by focusing on the accurate quantitation and standardization of Fragile X measurements.

PCR-based testing methods were examined and optimized to determine which protocols proved to be more robust for amplification of Fragile X repeat elements. We purchased a panel of Fragile X cell lines from the Coriell repository and have validated 5 of these cell lines for Fragile X testing using slab gel and capillary separation systems. We have applied statistical analysis to these measurements determining inter-gel variation as well as inter-lane variation for repeat elements of varying sizes. The effect of PCR amplification was also measured by conducting the amplification experiments in triplicate. We concluded from these data that reproducibility is independent of size but may depend on other factors such as DNA purity, concentration, and interruption of the repeat units. Recently, a PCR-based Fragile X assay was released in the marketplace. It is expected that some of the clinical laboratories will shift from their current assay systems (primarily home-brew) to this assay system. Future plans call for the evaluation of measurement sensitivity and variability of this new system in comparison to our current validated assay system. We will also maintain our collaborations with the clinical diagnostics community, providing them with accurate measurements and standards to aid in the development of guidelines to ensure accurate measurement for diseases associated with triplet repeats. The results of this research will be presented at the "Annual Clinical Genetics Meeting" in March 2001.



Left: Gel image of fluorescently labeled fragile X gene products from 5 individuals with normal and pre-mutation alleles. Right: Electropherogram of the size separations between these alleles, where 29 and 31 triplet repeats are clearly resolved by GeneScan ™ analysis.

Designing and Screening Small Molecules that Target RNA

J.P. Marino, J.T. Stivers (831/CARB), K.A. Lacourciere, and M. Rist (UMBI/ CARB)

Details provided in the **Bio-Molecules and Materials** section.

Telomere Quantitation for Tissue Engineering

P.E. Barker (831) and C. Jackson-Cook (Medical College of Virginia)

Details provided in the **Bio-Molecules and Materials** section.

Biomarkers for Quality Assurance and Quality Control of Tissue-Engineered Skin

H. Rodriguez, P.E. Barker, C. O'Connell, and M. Dizdar (831)

Details provided in the **Bio-Molecules and Materials** section.